Novel Frizzled-4 Gene Mutations in Chinese Patients With Familial Exudative Vitreoretinopathy

Li-Yun Jia, PhD; Xiao-Xin Li, PhD; Wen-Zhen Yu, PhD; Wo-tan Zeng, BSc; Chen Liang, BSc

Objectives: To search for mutations in the Frizzled-4 gene (FZD4) in Chinese patients with familial exudative vitreoretinopathy (FEVR) and to delineate the mutation-associated clinical features.

Methods: Forty-eight Chinese patients with FEVR and 100 unrelated control subjects were recruited and had complete ophthalmic examinations performed. The coding regions of FZD4 were screened for mutations by polymerase chain reaction and direct sequencing. Multiple sequence alignment was conducted to evaluate the conservation of residues among different FZD4 homologs and the human Frizzled family. Genotype-phenotype correlations were also analyzed.

Results: Twelve putative disease-causing mutations were identified in total, 9 of which were novel: 1 deletion (P14fsX57), 1 nonsense mutation (S491X), and 7 missense mutations (G22E, E180K, T237R, R253C, F328S, A339T, and D470N). Three reported FZD4 mutations were also detected: H69Y, M105V, and W496X. Remarkably, 2 patients who harbored compound heterozygous mutations (H69Y with E180K or W496X) had a more severe ocular phenotype than carriers of a single H69Y mutation.

Conclusions: FZD4 mutations were responsible for FEVR in 15 of 48 Chinese patients (31.3%) in this study, similar to other ethnic groups. This study supports the highly polymorphic nature of FZD4 with a differential mutation profile in the Chinese population.

Clinical Relevance: The profile of the mutations obtained in FZD4 further illustrates the complexity of FEVR and provides a better understanding of the genotype-phenotype correlations.

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naling activity and a single missense mutation caused a moderate level of reduction. However, whether the mutations could be used to predict the severity of the disease has yet to be reported. Thus, establishment of genotype-phenotype correlations could offer additional information for a more accurate prognosis, prenatal diagnosis, and genetic counseling.

To our knowledge, there is no report on FZD4 mutations in Chinese patients with FEVR. Mutation screening in FZD4 in our Chinese patients with FEVR could therefore further enrich the spectrum and frequency of mutations in FZD4-caused FEVR.

**METHODS**

**STUDY SUBJECTS**

Forty-eight Chinese patients with FEVR (37 familial and 11 sporadic) and their family members were recruited in the Department of Ophthalmology, Peking University People’s Hospital, Beijing, China. The diagnosis of FEVR was based on the presence of at least 1 of the typical clinical signs: peripheral retinal avascularization with abnormal retinal vascular formation, severe retinal exudates, retinal neovascularization, peripheral fibrovascular mass, macular ectopia, retinal folds, retinal detachment, or vitreous hemorrhage. Fundus fluorescein angiography was performed in selected cases to confirm the diagnosis. Unrelated control subjects were also recruited from the Department of Ophthalmology, Peking University People’s Hospital for conditions such as senile cataract, floaters, and itchy eyes. They were given the same ophthalmic examinations and were diagnosed as not having FEVR or other major eye diseases. The study protocol was approved by the Ethics Committee for Human Research of Peking University People’s Hospital and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all study subjects.

**FZD4 SCREENING**

Genomic DNA was extracted from 200 µL of whole blood using a Qiamp Blood Kit (Qiagen, Hilden, Germany). Two coding exons and adjacent sequences of FZD4 were screened by polymerase chain reaction followed by direct DNA sequencing with the BigDye Terminator DNA sequencing kit on a 3130XL analyzer (Applied Biosystems, Foster City, California), using the same set of primers described by Kondo et al.23 The reference sequence of FZD4 (GenBank NM_012193.2) was used for the identification of variations. The first nucleotide (A) of the initiation code (ATG) was defined as +1.

**MULTIPLE SEQUENCE ALIGNMENT**

Alignment of the novel FZD4 mutations was performed by ClustalX 1.83 software with default parameters (http://biowininformatics.unc.edu/software/opensource/index.htm). The following protein sequences were used for the alignment figure: human Frizzled-4 (GenBank NP_036325), Mus musculus (GenBank NP_032081), Rattus norvegicus (GenBank NP_072145), Gallus gallus (GenBank Q91A05), Xenopus laevis (GenBank Q9PT62), Danio rerio (GenBank NP_571222), human Frizzled-1 (GenBank NP_003496), human Frizzled-2 (GenBank NP_001457), human Frizzled-3 (GenBank NP_059108), human Frizzled-5 (GenBank NP_003499), human Frizzled-6 (GenBank NP_003497), human Frizzled-7 (GenBank NP_003498), human Frizzled-8 (GenBank NP_114072), human Frizzled-9 (GenBank NP_003499), and human Frizzled-10 (GenBank NP_009128).

**RESULTS**

**FZD4 SCREENING**

In total, 16 heterozygous variants were identified, 13 of which were novel. Twelve variations, P14fsX57, G22E, H69Y, M105V, E180K, T237R, R253C, F328S, A339T, D470N, S491X, and W496X, that cosegregated with the disease in the families and were not found in 100 control subjects were considered disease-causing mutations. The characteristics of these mutations are presented in Table 1 and Figures 1, 2, 3, and 4.

**FRAMESHIFT MUTATION**

A 10-base pair deletion in exon 1 (c.39-49delCCC-GGGGCG) was identified in a 4-generation family with FEVR. This mutation caused a frameshift after codon 14 and a premature termination at codon 57 (P14fsX57).

**NONSENSE MUTATIONS**

Two nonsense mutations (S491X and W496X) were detected in exon 2. The S491X mutation resulted from a C→A transversion in nucleotide 1472, where the highly conserved serine residue is located in the transmembrane domain. The effect of this premature termination would result in a shortened protein of 490 amino acids instead of the 537 amino acids in the wild-type protein. The W496X mutation resulted from a G→A transition in nucleotide 1488, where the highly conserved tryptophan residue is located in the beginning of the C-terminal cytoplasmic tail, adjacent to the conserved Lys-Thr-X-X-Trp motif. The effect of this premature termination would result in a shortened protein of 495 amino acids. Remarkably, this family harbored another mutation, H69Y.

**MISSENSE MUTATIONS**

Nine missense mutations were identified. In exon 1, 2 mutations (G22E and H69Y) were identified. The G22E mutation was found in a patient with sporadic FEVR, while H69Y was identified in 4 families with FEVR. In exon 2, 7 mutations were identified. The M105V and D470N mutations were identified in 2 families with FEVR, while E180K, T237R, R253C, F328S, and A339T were identified in 5 different patients, each of whom had a family history of FEVR.

**OTHER VARIANTS**

In addition to the mutations described earlier, a nonsynonymous change, E166K, was found in a control individual. Two synonymous changes, Q54Q and V517V, were also detected in 2 control individuals. Moreover, a polymorphism was identified in intron 1 (IVS1 + 8C→T) in a control individual.
A highly variable phenotype was noted in the Chinese family with the P14fsX57 mutation. In 2 of these affected individuals, FEVR could only be diagnosed by fundus fluorescein angiography without any other clinical problems, whereas other affected individuals had a more severe range of phenotypes including macular folds and retinal detachments (Table 2, Figure 3, and Figure 4).

The G22E mutation was detected in an 8-month-old girl with sporadic FEVR. She was born at full term and at normal weight. Bilateral nystagmus was noted at age 3 months. Examination showed bilateral retrolental fibrosas and total retinal detachment without systemic manifestations related to this mutation. Screening of NDP did not show any disease-associated polymorphisms (data not shown). No sequence change was found in either parent, suggesting that G22E is a sporadic mutation.

A compound heterozygous patient who harbored 2 FZD4 missense mutations, H69Y and E180K, was a 35-year-old woman showing extensive vitreoretinal traction distorting the retinal vessels emerging from the optic disc in the right eye and a falciform retinal fold in the left eye. Genotypes of the parents showed 1 of the 2 mutations in each: the father was heterozygous for H69Y and the mother was heterozygous for E180K. The parents showed no signs of retinopathy. However, their 16-year-old son who showed retinal traction at the posterior pole and acute-angled vascular branches in both eyes carried only a single H69Y mutation (Figure 4A).

Another compound heterozygous patient was a 6-month-old boy carrying H69Y and W496X. He had leukokoria in the right eye and severely extensive vitreo-retinal traction in the left eye. His mother carried a single H69Y mutation and showed mild retinal traction with macular ectopia in the left eye and straightening of vessels at the posterior pole in both eyes. His maternal grandfather and uncle with a single H69Y mutation were emmetropic and appeared clinically healthy. His father, who carried a single W496X mutation, had only a sign of peripheral retina nonperfusion observed by fundus fluorescein angiography in both eyes (Figure 4B). Patients with compound heterozygous mutations had more severe phenotypes than carriers of single mutations in both families.

The T237R mutation was detected in a 10-month-old boy with a retinal fold in the right eye and peripheral retinal avascularization in both eyes. The father, who carried the mutation, was emmetropic and appeared clinically healthy. The proband with the R253C mutation was a 6-month-old boy who showed bilateral vitreous opacity and retinal exudates. The mother had bilateral peripheral avascularization with a typical scalloped border. Furthermore, the proband’s maternal uncle was amblyopic with a history of retinal detachment. The proband with the F328S mutation was a 9-month-old girl who had bilateral retinal folds resembling persistent hyperplastic primary vitreous. The father, who carried the mutation, showed only myopia and myopia-associated visual deterioration but no signs of retinopathy. Although both parents did not have any ocular abnormalities, her patrilineal nephew had a diagnosis of FEVR because of bilateral retinal folds at age 2 years. The patient with the A339T mutation was noted to have classic features of FEVR. Her mother had dragged discs and temporal sectors of pigment change typical of FEVR. The D470N mutation was detected in 2 families. The proband of one family was a 4-year-old girl. Her left eye showed areas of preretinal fibrosis, and both eyes showed...
**Figure 1.** Protein sequence alignment of human Frizzled-4 with Frizzled-4 homologs from other species including mouse, rat, chicken, frog, and zebra fish. The novel mutations G22E, E180K, and D470N are indicated. Frizzled-4 proteins and the human Frizzled family proteins. Twenty amino acid residues surrounding each mutation are shown. The novel mutations G22E, E180K, R253C, F328S, A339T, and D470N are indicated.

We screened 48 unrelated patients with FEVR to determine the types and frequencies of mutations in FZD4 and identified 12 different disease-causing mutations, 9 of which were novel. All of these mutations were responsible for adFEVR. Our data indicated that FZD4 mutations were responsible for 15 of 48 cases of FEVR (31.3%) (95% confidence interval, 20.0%-45.3%) in Chinese pa-

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human FZD2</td>
<td>FARLWIL TWSVLCCASTFFTVTTYLVDMQRFRYPERPIIF</td>
</tr>
<tr>
<td>Mouse FZD1</td>
<td>FSR TWIGIWSVLCCASTLFTVLTYLVDMRRFSYPERPIIF</td>
</tr>
<tr>
<td>Frog FZD4</td>
<td>FTDIWMAVWASLCFISTFTVLTFLIDSSRFCYPERPIIF</td>
</tr>
<tr>
<td>Chicken FZD4</td>
<td>FTDIWMAVWASLCFISTFTVLTFLIDSSRFSYPERPIIF</td>
</tr>
<tr>
<td>Rat FZD4</td>
<td>FTDIWMAVWASLCFISTFTVLTFLIDSSRFSYPERPIIF</td>
</tr>
<tr>
<td>Mouse FZD4</td>
<td>FTDIWMAVWASLCFISTFTVLTFLIDSSRFSYPERPIIF</td>
</tr>
<tr>
<td>Zebra fish FZD4</td>
<td>FTDIWMAVWASLCFISTFTVLTFLIDSSRFSYPERPIIF</td>
</tr>
<tr>
<td>Human FZD10</td>
<td>FAV V WLAIWAVLCFFSSAFTVLTFLIDPARFRYPERPIIF</td>
</tr>
<tr>
<td>Human FZD8</td>
<td>TVSTFLIDMERFKYPERPIIFLSACYLFVSVGYLVRLVAG</td>
</tr>
<tr>
<td>Human FZD9</td>
<td>TVL TFLLEPHRFQYPERPIIFLSMCYNVYSLAFLIRAVAG</td>
</tr>
<tr>
<td>Human FZD10</td>
<td>TVLTLIDPARFYP FIMLXYCVSCYSVGLLGYFLG</td>
</tr>
<tr>
<td>Human FZD4</td>
<td>NW ALFRYSADDS --------------------------N M</td>
</tr>
<tr>
<td>Mouse FZD3</td>
<td>T F L TFLIDVTRFRYPERPIIFLYAVCYMMVSLIFFI</td>
</tr>
<tr>
<td>Mouse FZD9</td>
<td>MAV APLRGA- - - - - - - - - - - - - LLL WQLLAAGGAAL- - - -</td>
</tr>
<tr>
<td>Human FZD10</td>
<td>MQRPGPR- - - - - - - - - - - - - LWLLOVNSCA- - - -</td>
</tr>
</tbody>
</table>

**COMMENT**

We screened 48 unrelated patients with FEVR to determine the types and frequencies of mutations in FZD4 and identified 12 different disease-causing mutations, 9 of which were novel. All of these mutations were responsible for adFEVR. Our data indicated that FZD4 mutations were responsible for 15 of 48 cases of FEVR (31.3%) (95% confidence interval, 20.0%-45.3%) in Chinese pa-


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tients, which is comparable to previous reports in other ethnic groups (20%-40%).

Mutations of FZD4 are known to cause inherited as well as sporadic FEVR in various ethnic populations. Frizzled-4 is a cell surface receptor of Wnt signaling, which activates the Wnt canonical pathway involving β-catenin stabilization and T-cell factor–dependent gene transcription in the neural retina, ciliary margin, and other ocular structures. FZD4 is clearly vital for normal development of the intraretinal vasculature in mice and humans. Autosomal dominant FEVR was found to be associated with apparent loss of function of the Frizzled-4 protein.

In our study, 3 of 12 disease-causing mutations were predicted to result in truncation of the Frizzled-4 protein.
tein, the proportions ranging from about 90% deletion for P14fsX57 to 8% for W496X. These mutations in Frizzled-4 resulted in premature termination codons, which might cause nonsense-mediated messenger RNA decay-induced haploinsufficiency. However, the precise effect of premature termination codons on Frizzled-4 has not been investigated; therefore, it is still unknown whether the mutant protein would be created. The P14fsX57 deletion is located in the N-terminal region of the Frizzled-4 signal peptide (Figure 2). Therefore, FZD4 messenger RNAs that contain truncating mutations could be degraded and would not be translated. The other nonsense mutations (S491X and W496X) were located within the last exon, which would result in a truncated protein. The W496X mutation was also found in Dutch patients with FEVR. Further experiments are needed to determine the actual effects of these mutations on the Frizzled-4 protein.

One patient with sporadic FEVR who had the novel G22E mutation had the typical finding of FEVR without systemic manifestations. The glycine residue is located in the N-terminal region of the Frizzled-4 signal peptide (Figure 2), directing the protein into the plasma membrane as predicted by SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The missense change is a nonconservative substitution and the residue is well conserved among the orthologs (Figure 1). The missense change G22E may alter the translocation of Frizzled-4 protein and affect the retinal vascularization that leads to FEVR. The G22E mutation was not detected in the parents, indicating that it is de novo. The region of the Frizzled-4 signal peptide is not a mutation hot spot; to our knowledge, only P33S and G36D have been reported to be related to FEVR. Hence, the founder mutation G22E is the third one in this region.

Three missense changes (H69Y, M105V, and E180K) were located within the extracellular cysteine-rich domain (CRD) of Frizzled-4 (Figure 2), and these missense changes are nonconservative substitutions. All of the residues are well conserved among the orthologs of Frizzled-4. All Frizzled receptors have an extracellular CRD with 10 invariant cysteines for the binding of the Wnt proteins. Although the overall sequence identity of the CRD ranges from 30% to 50% among Frizzled receptors (Figure 1), they adopt the same 3-dimensional crystal structure because of the conserved cysteines.
remaining variability among the 10 human Frizzled receptor CRDs is thought to underlie their different Wnt binding specificities. This may explain why the mutated residues in the CRD are highly conserved among orthologs but less conserved among the other human Frizzled receptors.

The T237R mutation occurred at a highly conserved threonine residue located in the first transmembrane region of FZD4. The R253C, F328S, and A339T mutations were located in the first or third cytoplasmic loops, in which arginine at codon 253, phenylalanine at codon 328, and alanine at codon 339 were highly conserved across all members of the Frizzled family as well as 6 different species of FZD4, which suggests that substitution in these positions may compromise protein function and lead to a disease phenotype. The D470N mutation occurs at a highly conserved aspartic acid residue located in the extracellular loop. Although the pathogenic effects of all of these missense mutations have not been proven, these data suggest that they are likely to be pathogenic (Figure 1 and Figure 2).

Interestingly, we found 2 patients who harbored the H69Y mutation with a second mutation, E180K or W496X (Figure 4). Highly conserved codon 69 was located in the CRD. However, H69Y was also found in Japanese healthy individuals and might be considered a polymorphism. Alternatively, H69Y might be pathogenic with low penetrance because this change was found with higher frequency in patients with FEVR than in the normal population. Consistent with this assumption, the 2 patients with compound heterozygous FZD4 mutations presented a more severe phenotype than those who carried a single mutation. Moreover, Kondo et al previously reported that 1 patient with adFEVR who had 2 FZD4 mutations, G488D and H69Y, had a more severe ocular phenotype compared with a patient who harbored G488D alone. Furthermore, Qin et al showed that H69Y displayed Wnt signaling reductions and decreased the binding affinity to norrin compared with wild type in vitro. The H69Y mutation cosegregated with the disease in the 2 families with adFEVR in this study and 1 Japanese family with adFEVR, suggesting a disease-causing nature of the H69Y mutation. Notably, Shastry and Trese reported that a factor V mutation cosegregated with the FZD4 L501Sx533 mutation in a patient with adFEVR.

In addition, Qin et al described a patient who harbored both the R417Q mutation in FZD4 and the R444C mutation in LRP5. These studies suggest a synergistic effect of the 2 mutations in the independent FEVR-causing genes, indicating a possible digenic form of inheritance. Given these facts, we speculated that some patients with FEVR may have a complex genetic trait rather than a simple monogenic inheritance, contributing to the clinical variability in patients with FEVR. Further investigations are required to evaluate the functional significance of these sequence changes.

Previous studies showed that 3 FZD4 mutations (M105V, M157V, and M493-W494del) were unable to activate either Wnt or a norrin-dependent signaling pathway. Recently, Qin et al showed that a nonsense mutation (W319X) in FZD4 completely abolished Wnt signaling activity, while missense mutations (M105V, R417Q, and R444C) of the 2 mutations in the independent FEVR-causing FZD4 played Wnt signaling reductions and decreased the binding specificities. This may explain why the mutations in the extracellular loop are thought to underlie their different Wnt

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**Figure 4.** Schematic pedigrees of the family with FZD4 mutations H69Y and E180K (A) and of the family with FZD4 mutations H69Y and W496X (B). Arrows indicate probands; open symbols, clinically unaffected; solid symbols, clinically affected; and circles inside symbols, asymptomatic for familial exudative vitreoretinopathy. C, Fundus images of the proband of family 14 (II-3) with H69Y and W496X, showing extensive vitreoretinal traction distorting the retinal vessels emerging from the optic disc and producing a retinal fold extending across the posterior pole to the temporal periphery in the right eye and a falciform retinal fold in the left eye (top left and right images). D, Fundus images of individual II-3 in family 15 with only the H69Y mutation showing mild vitreoretinal traction with macular ectopia in the right eye and straightening of vessels at the posterior pole and acute-angle vascular branching in both eyes (bottom left and right images).
and H69Y) caused a moderate level of reduction. However, some heterozygous patients with a mutation in FZD4 did not show any sign of a FEVR phenotype, as exemplified by the father of a patient with the T237R mutation in this study. Therefore, a 50% reduction of FZD4 activity alone might not be sufficient to trigger adFEVR in some cases. One of the nonsense mutations (L501fsX533) was experimentally shown to be absent from the plasma membrane, suggesting a dominant-negative effect. Therefore, we suspected that the dominant-negative effect may be the cause in some cases of adFEVR; however, this remains to be proven in functional studies of the mutant proteins.

The clinical features presented in most of our patients with FEVR who had FZD4 mutations were comparable to classical descriptions of FEVR and to the phenotypes linked to FEVR loci. As a consequence, we were not able to distinguish FEVR features specific to FZD4 or genotype-phenotype correlations among different mutations even in widely variable phenotypes observed within the same family. It is of interest to speculate why these patients with FEVR have defects only in the development of the retinal vasculature as FZD4 is widely expressed throughout the body. Recently, Ye et al showed that norrin/Frizzled-4/LRP5 signaling regulated the interactions of endothelial cells and mural cells and is essential for vascular integrity in the retina and cerebellum. FZD4 is active throughout development and in the adult vasculature, while inappropriate activation of FZD4 results in severe vascular disorganization.

In summary, 9 novel mutations broadened the existing spectrum of FZD4 mutations, especially in Chinese patients with FEVR. At present, results of genetic testing should be used with caution for prognosis or counseling. The profile of the mutations obtained in our study further illustrates the complexity of this disease and provides a better understanding of the spectrum and frequencies of FZD4 mutations in FEVR as well as the genotype-phenotype correlations.

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Correspondence: Xiao-Xin Li, PhD, Department of Ophthalmology, Peking University People’s Hospital, Beijing 100073, China (lixirxiaoxineye@gmail.com).

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Additional Contributions: We thank all of the patients and families for their cooperation in this study. Calvin Chi-Pui Pang, BSc, DPhil, Department of Ophthalmology, Peking University People’s Hospital, Beijing 100073, China (lixirxiaoxineye@gmail.com).

Table 2. Mutations in the FZD4 Gene and the Associated Clinical Findings

<table>
<thead>
<tr>
<th>Patient No./Sex/Age</th>
<th>Sequence Change</th>
<th>Visual Acuity (Refraction)</th>
<th>Clinical Findings</th>
</tr>
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<tbody>
<tr>
<td>1 III-5/F/15 y</td>
<td>P141fsX57</td>
<td>0.16 (−5.0 D) OD; 0.1 (−4.5 D) OS</td>
<td>Avascular retina, dragged macula, RLF, and RD OU</td>
</tr>
<tr>
<td>2 III-1/F/8 mo</td>
<td>G22E</td>
<td>Light perception OU</td>
<td>Nystagmus, RLF, and RD OU</td>
</tr>
<tr>
<td>3 III-2/M/8 mo</td>
<td>H69Y</td>
<td>Follow a moving object OU</td>
<td>Retinal folds and PHPV OU</td>
</tr>
<tr>
<td>4 III-1/F/4 y</td>
<td>H69Y</td>
<td>0.2 (NC) OD; 0.1 (NC) OS</td>
<td>Retinal folds and macular ectopia</td>
</tr>
<tr>
<td>5 II-1/F/3 y</td>
<td>M105V</td>
<td>Follow a moving object OU</td>
<td>Retinal folds, macular ectopia, and RD OU</td>
</tr>
<tr>
<td>6 III-1/F/18 y</td>
<td>M105V</td>
<td>0.3 (−6.5 D) OD; 0.6 (−4.0 D) OS</td>
<td>Retinal vascular tortuosity, exudates, and avascularization OU</td>
</tr>
<tr>
<td>7 III-2/M/9 y</td>
<td>T237R</td>
<td>HM (NC) OD; 0.1 (NC) OS</td>
<td>Preretinal fibrosis, peripheral nonperfusion, and RNV OD; peripheral nonperfusion and RNV OS</td>
</tr>
</tbody>
</table>

Abbreviations: D, diopters; HM: hand motions; NC, not corrected; PHPV, persistent hyperplastic primary vitreous; RD, retinal detachment; RLF, retrolental fibroplasia; RNV, retinal neovascularization.

Family and patient numbers refer to those shown in Figure 4.
REFERENCES


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